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Antibacterial and Anti-Adherent Activity of Great Mullein (Verbascum Thapsus L.) Ethanolic Extract on in Vitro Biofilm Formation of Three Oral Streptococci

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Abstract

Background: *Verbascum thapsus (VT) is a medicinal plant that chemical constituents of it revealed the presence of biologically active compounds with antibacterial properties. The aim of the present study was to evaluate the antimicrobial and anti-adherent activities of ethanol extract of (VT) against of three oral streptococci in vitro.*

Methods*: In this study, biofilm formation of S. mutans 1683ATCC35.668, S. sanguinis 1449CIP53.15 and S. salivarius 1448 CIP55.128 with ethanol extract of VT was tested using Micro-dilution assay and microtitre plate assay.*

Results*: Results showed that the biofilm formation of three oral streptococci with ethanol extract (leaves and root) of VT was significantly lower than that of the control group without ethanol extract of VT. Meanwhile, the reduction degree was correlated to the concentration of ethanol extract of VT positively.*

Conclusions*: These results suggest that antimicrobial activity of ethanol extract of VT against three oral streptococci. VT extracts have inhibitory effects on biofilm formation of oral streptococci as the reduction of bacterial growth and reduction of biofilm formation ability.*

Keywords: Verbascum thapsus, Ethanol extract, Oral streptococci, Antimicrobial activity, Biofilm.
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Introduction

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix.[1] The oral microbial flora comprises one of the most diverse human-associated biofilms. Its development is heavily influenced by oral streptococci, which are considered the main group of early colonizers.[2] Numerous studies have shown that these bacteria are capable of adhering and forming biofilm on oral cavity and tooth. The oral streptococci, especially Streptococcus mutans and Streptococcus sanguinis have often been associated with dental caries in humans and another one such as Streptococcus salivarius has been linked with the disease or absence of it.[3] Streptococcus mutans is a facultative anaerobic, Gram-positive coccus-shaped bacterium commonly found in the human oral cavity and is a significant contributor to tooth decay[4] and Streptococcus sanguinis mostly found in dental plaque and cavities within the healthy oral mouth.[5] Streptococcus salivarius also is known to be a pioneer colonizer of oral surfaces. Although, S. salivarius is a normal member of the human oral microbiome that is an uncommon cause of invasive infections but when isolated from blood cultures, this viridans streptococcus is often disregarded as a contaminant. Meningitis is a rare but increasingly reported infection caused by S. salivarius.[6] Verbascum thapsus (Schrophulariaceae) better known as Great Mullein is a medicinal, native to Europe, northern Africa and Asia, and introduced in the Americas and Australia.[7] It is a hairy biennial plant that can grow to 2 m or more tall. Its small yellow flowers are densely grouped on a tall stem, which bolts from a large rosette of leaves.[8] Previous studies regarding the chemical constituents of VT revealed the presence of biologically active compounds, such as flavonoids,[9] iridoid glucosides,[10] phenylethanoid, lignin glycosides[11] and sesquiterpenes.[10] Phytochemicals also reports that leaves of VT are rich in iridoid glycosides, flavonoids, phenylethanoid glycosides and saponins and they may be responsible for the biological activities.[12] Antiviral, antimicrobial, antioxidant, anti-inflammatory and cytotoxic activities of Verbascum species have been previously reviewed.[12] VT leaves and flowers are reported to have expectorant, mucolytic and demulcent properties[13] and traditionally, VT is used as ethnomedicine for the treatment of inflammatory disease, asthma, spasmodic coughs, and migraine. The seeds are reported to be aphrodiasic and narcotic in nature.[14] Their phenolic constituents are considered to be responsible for the anti-inflammatory and antimicrobial activity of the herb. VT flowers are highly valued herbal drugs used in the treatment of inflammation, asthma, spasmodic coughs and other respiratory tract diseases.[15] Meanwhile, the plant does contain coumarins and other toxins so it should be used wisely.[14] Based on McCutcheon et al., 1995[16] reported, extracts of VT revealed antiviral activity against Herpes virus type 1. VT has been investigated for their antibacterial activity through in-vitro and in-vivo tests and Turker et al 2001[17] showed VT extract possesses compounds with antibacterial properties. Panchal et al[18] reported that extract of VT flowers in olive oil, had antibacterial activity against Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus. This activity was attributed to the saponins. As it was stated in a report by Dulger et al[19] extracts obtained from three Verbascum species (Verbascum olympicum, Verbascum prusianum and Verbascum bombyciferum) revealed antibacterial activity against the Gr (+) bacteria. According to previous study by

Ozcan et al[20] the various extracts of Verbascum pinetorum exhibited antimicrobial activity in different values on the Gr (+) and Gr (-) bacteria. Extracts of Verbascum gypsicola and Verbascum sinuatum also have demonstrated antimicrobial activities.[21] Kahraman et al[22] also reported that Verbascum mucronatum Lam. and Verbascum olympicum showed an antibacterial activity against Gr (+) bacteria, and staphylococcus aureus as well as Verbascum latisepalum showed an antifungal activity against Candida krusei. However, to date, the effect of ethanol extract of VT on oral streptococci biofilm formation has not been reported, in this study, we investigated the effects of ethanol extract of VT against biofilm-forming of three oral streptococci in vitro.

Materials and Methods

Fresh aerial growing parts and root of V. thapsus were collected during the period of June-July 2014 from Jangal-e Abr, Shahroud, Iran. Dr. Esmail Babakhanzadeh Sajirani from the Department of Horticulture, Applied Agricultural Science, Education Center, Shahroud, in Iran located and identified the plants.

Plant materials (leaves, flowers, and roots) were dried in shade and ground to fine powders. In total, 20 g of each dry powdered plant material were soaked in 200 ml ethanol (95%) and kept aside for 2 days, then filtered with Whatman filter paper no.1 and the filtrate was evaporated under vacuum in a rotary evaporator at 55°C. The extract yield, which was sticky and black, was stored in labeled sterile crew-capped bottles at 20°C.

The various extracts of VT were individually tested on three oral Streptococci strains, including S. mutans 1683 ATCC35.668, S. sanguinis 1449CIP53.15, and S. salivarius 1448 CIP55.128. These bacteria were cultured overnight at 37°C.

The broth microdilution method was used to determine the minimal inhibitory concentrations (MIC) and the minimum bactericidal concentration (MBC), according to Shayan and Saeidi,[23] guidelines. All tests were first dissolved in DMSO (Sigma-Aldrich)-Mueller Hinton Broth (MHB; Sigma-Aldrich). In brief, serial twofold dilutions of the plant extracts were put in a 96-well microtiter plate and the turbidity was adjusted to 0.5 McFarland and diluted to obtain a final turbidity in wells of approximately 1 × 106 CFU/mL. An amount of 70 µL of plant extract solution (prepared by dissolving a plant extract in DMSO) 70 µL of bacterial inoculum, which were placed into wells of microtiter plate, and 70 µl of MHB were added and incubated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18-24 h. The color change was then visually assessed. The lowest concentration at which the color change occurred was taken as the MIC value. The average of three values was calculated, providing the MIC and MBC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity. The MBC was defined as the lowest concentration of the extract at which the incubated microorganism was completely killed.

To analyze the biofilm formation by three isolated staphylococci, the method described by[24] was used with modifications. Eight wells of a sterile 96-well polystyrene plate were filled with 200 µl of the appropriate medium (Muller-Hinton Broth) as negative control, six wells were filled with 190 µl of the medium with 10 µl of overnight bacterial culture as positive control. A quantity of 175 µl of the medium + 25 µl of (leaves, flowers, and roots) VT extracts were added into 12 wells, and nine wells were filled with 165 µl of the medium + 10 µl of each strain of selected bacterium+25 µl of VT extracts. The microtiter plates were then incubated at 37°C for 24 h. After performing the procedure described above, the microplate was covered and incubated aerobically for 24 h at 35°C. The content of the plate was then poured off and the wells were washed three times with 300 µl of sterile physiological saline (PBS, pH=7.2) containing 3% NaCl or 0.5% glucose. The remaining attached bacteria were fixed with 200 µl of 99% methanol per well, and after 20 min all of the wells were emptied and left to dry. Then, each well was stained for 5 min with 250 µl of 2% crystal violet. Excess stain was rinsed off by washing the plate slowly with distilled water. After 15 min, when the plate was air dried, the dye bound to the adherent cells was re-solubilized with 200 µl of 33% (v/v) glacial acetic acid per well. The O.D. of each well was measured at 545 nm by using an automated Eliza counter Stat Fax 2100 Microplate Reader. Based on the O.D. produced by bacterial films, strains were classified into the following categories: no biofilm producers, weak, moderate, or strong biofilm producers, as previously described (25). In brief, the cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. Strains were classified as follows: O.D.≤O.D.c=no biofilm producer, O.D.c<O.D.≤(2×O.D.c)= weak biofilm producer, (2×O.D.c)<O.D.≤(4×O.D.c)= moderate biofilm producer and (4×O.D.c)<O.D.=strong biofilm producer.

The assay was conducted in paired triplicates, and the t-test was used for statistical comparisons between groups. The level of statistical significance was set at a P≤0.05.

Results

In this study, the antimicrobial activities of ethanolic extracts obtained from the leaves, flowers, and roots of VT against three standard strains of oral staphylococci were examined. The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of the VT extracts against these strains are given in Table 1. From the results of the MIC, it was observed that almost all tested bacterial strains were sensitive towards the ethanolic extracts of VT. The highest MIC for the leaf extract of VT was 0.50 mg/ml and three strains were inhibited at this concentration, while the highest MIC for the VT flower ethanolic extract that inhibited S. mutans, S. sanguinis, and S. salivarius were 1, 0.25, and 0.5 mg/ml, respectively. The best MIC concentration for VT root extract was 0.25 mg/ml, which inhibited S. sanguinis and S. salivarius; S. mutans was inhibited at 1 mg/ml root extract of VT. The highest MBC for VT leaves was 1 mg/ml and the whole three strains were lost at this level of concentration. However, the best MBC for root and flower extracts was 2 mg/ml and S. mutans was destroyed at this level of concentration. S. sanguinis and S. salivarius were destroyed by

Table 1. Antibacterial activity of ethanolic extract of VT extract against three oral streptococci strains

Bacteria strains	Verbascum thapsus L.		
	Leaf extract (mg/ml) MIC/MBC	Flower extract (mg/ml) MIC/MBC	Root extract (mg/ml) MIC/MBC
S. mutans 1683ATCC35.668	0.50/1	1/2	1/2
S. sanguinis 1449CIP53.15	0.50/1	0.25/0.50	0.25/0.50
S. salivarius 1448 CIP55.128	0.50/1	0.50/1	0.25/0.50

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values are given as mg/ml for extract

0.50 mg/ml of root and flower extracts of VT, and 1 mg/ml of flower extract and 0.50 mg/ml of VT root extract eradicated S. salivarius (Table 1).

To investigate whether ethanol extracts of VT affects biofilm formation of S. mutans 1683 ATCC35.668, S. sanguinis 1449 CIP53.15, and S. salivarius 1448 CIP55.128 we monitored the biofilm formation of these standard strains of oral staphylococci using microtitre plate assay. The results of in vitro anti-biofilm activity of VT ethanolic extract are presented in Table 2. The control group without ethanol extract of VT formed strong biofilm after 24 h incubation at 37°C. However, strains of the experimental groups with different concentrations of ethanol extract of VT (leaves, flowers, and roots) formed weaker biofilm after crystal violet staining.

Table 2. Anti-biofilm activity of ethanolic extract from verbascum thapsus

Bacteria strains	VT extract			Positive Control
	Leaf	Flower	Root	
S. mutans 1683ATCC35.668	NB	NB	NB	S
S. sanguinis 1449CIP53.15	M	W	W	S
S. salivarius 1448 CIP55.128	M	NB	W	S

NB- No biofilm formation, M- Moderate biofilm formation, W- Weak biofilm formation and S-Strong biofilm formation

All ethanolic extract of VT (leaves, flowers, and roots) showed no biofilm formation of S. mutans 1683 ATCC35.668. Although, roots extract demonstrated moderate biofilm activities and flowers extract demonstrated weak biofilm activities, but the leaf extract of VT showed no biofilm formation of S. sanguinis 1449CIP53.15. To investigate the effect of ethanol extract of VT on biofilm formation of S. sanguinis 1449CIP53.15, we observed that the leaves and flowers of VT formed a significantly weak biofilm. Moreover, roots extract of VT demonstrated moderate biofilm activity.

Discussion

The results of the present study showed the potential of antimicrobial activity of VT against bacterial strains of three oral Streptococci. Moreover, the biofilm formation of these strains with ethanol extract of VT was significantly lower than the biofilm formation of positive control group. This degree of reduction also was positively correlated to the concentration of ethanol extract of VT. These findings reinforce the notion that VT extract possesses compounds with antimicrobial properties. Our findings confirmed the observations of some other researchers, which stated that some Verbascum species contain biologically active compounds with antimicrobial properties such as flavonoids, phenylethanoid and neolignan glycosides, saponins, and iridoid glycosides.[12] However, VT, similar to many other Verbascum species, has not been investigated in terms of anti-biofilm formation activity, but VT extract showed antibacterial activity against K. pneumonia, Staphylococcus aureus, Staphylococcus epidermidis, and E. coli.[17] Antimicrobial activities of V. qulebriu, V. blattaria, V. bombyciferum, V. chaixii, V. dumulosum, V. nigrum, V. olympicum, V. phlomoides, V. phoeniceum, V. roripifolium, V. sinaiticum, V. macrurum, and V. georgicum have been previously reported by.[22] In a study conducted by,[26] V. geopgicum showed an inhibitory effect on the growth of different species of bacteria, namely Bacillus amyloliquefaciens, B. subtilis, B. cereus, B. pumilus, B. megaterium, B. lentimorbus, B. licheniformis, Pseudomonas putida, and P. syringae; additionally, E. coli., V. mucronatum, and V. olympicum also showed an antibacterial activity against gram-positive bacteria, as well as S. aureus and V. latisepalum.[22] Here, we provided evidence that ethanol extract VT has inhibitory effect on biofilm formation of S. mutans, S. sanguinis, and S. salivarius. Biofilm formation of oral Streptococci, such as S. mutans, has an impact on human health and biofilm bacteria.[5] S. mutans is a primary bacterium involved in plaque formation and in the initiation of dental caries. Therefore, searching for new organic compounds to inhibit the biofilm formation of these bacteria has become a new strategy for clinical infection treatment.

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Conflict to Interest

The authors declared that they have no conflict of interest.

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